

Because the toxicity profile of ddC was different from that of AZT, we reasoned that their combination might yield improved anti-HIV activity with lower toxicity than either drug used alone, and in an extension of the same protocol, we treated an additional six patients with an alternating regimen of AZT and ddC (Yarchoan et al. 1988). An alternating (rather than simultaneous) regimen was utilized because it could be done quickly without the need for combination drug testing in animals. This regimen was well tolerated, and the five patients who completed 9 weeks had a mean increase of 48 CD4 cells/mm³ and tended to have a decrease in p24 antigen. This trial was the first reported study of a combination regimen of effective anti-HIV drugs. Subsequent studies confirmed that the AZT/ddC combination was well tolerated in children and adults (Bozzette and Richman 1990; Pizzo et al. 1990) and provided evidence that the combination of AZT and ddC, administered together, provided better virologic and immunologic activity than AZT alone. ddC (zalcitabine) was ultimately approved by the US FDA as an anti-HIV drug for use in combination with AZT on June 19, 1992. This did not occur until after ddI (didanosine) had been approved, largely because of ddC's relatively weak clinical activity and problematic toxicity profile.

1.6 Development of Didanosine (2',3'-Dideoxyinosine, ddI)

Our group next turned our attention to the purine analogues ddA and ddI. Both of these compounds are phosphorylated in cells to 2',3'-dideoxyadenosine-5'-triphosphate, which, like AZT-triphosphate and ddC-triphosphate, displayed activity against HIV RT (Ahluwalia et al. 1987, 1997; Cooney et al. 1987; Mitsuya and Broder 1986, 1987; Yarchoan et al. 1989a). Once converted to the 5'-triphosphate moiety, their cellular half-life is more than 12 h, compared to ~3 h for AZT 5'-triphosphate, and could thus potentially be administered only once or twice a day, as a major advantage in treating a chronic infectious disease. Because it seemed more straightforward, clinical development first focused on ddA, and a phase I study of intravenous ddA was initiated in February, 1988 (Yarchoan et al. 1989c).

A barrier to development of these compounds as oral agents was that they rapidly undergo solvolysis under acid conditions to form the free base and dideoxyribose (Cooney et al. 1987); because the stomach is an acid environment, this could reduce their oral absorption. Moreover, it was appreciated that adenine, a product of acid cleavage of ddA that would form in the stomach, could cause renal damage (Linblad et al. 1973); by contrast, the free base of ddI, hypoxanthine, did not have such toxicity. Also, it was recognized that ddA was rapidly converted to ddI in plasma, so that no matter which drug was administered, the form circulating in plasma would be largely ddI. In cells, ddI was then phosphorylated to ddA-5'-triphosphate. For these reasons, the focus of clinical development soon switched to ddI, and a phase I study of this compound was started in October of 1988. To address the problem of acid lability affecting oral absorption, the relatively simple approach was taken of administering ddI with antacids, and when given this way, adequate oral absorption was

attained (Hartman et al. 1990; Yarchoan et al. 1989c). As with ddC, the discovery of anti-HIV activity and preclinical development of these drugs was undertaken at the NCI; during its early development, ddI was licensed to Bristol-Myers Squibb Co., which became a partner in its development.

The NCI phase I trial of ddI, which involved 26 patients receiving the drug for up to 42 weeks, showed evidence of anti-HIV activity, especially at the higher doses of 3.2 mg/kg every 12 h orally. In particular, patients had increases in their CD4 cells, improved immunologic function, weight gain, and substantial decreases in HIV p24 antigen (Yarchoan et al. 1990b, 1989b). The principal toxicities were peripheral neuropathy, liver function abnormalities, and rare cases of pancreatitis. Soon after initiation of the NCI study, two others were initiated, one testing it once daily and the other twice daily, and ddI in these trials was found to have similar activity and toxicity profiles to that reported in the initial NCI trial (Cooley et al. 1990; Lambert et al. 1990). In addition, a phase I/II study initiated in children at the NCI showed in addition that it could reverse HIV-associated developmental abnormalities (Butler et al. 1991). Based on these results, several randomized trials were initiated; these trials demonstrated that patients who had been receiving AZT (zidovudine) did better if they were switched to ddI (didanosine) (Delta Coordinating Committee 1996; Kahn et al. 1992; Spruance et al. 1994). Meanwhile, on October 9, 1991, even before these randomized trials were completed, the US FDA approved ddI for both children and adults with advanced AIDS based largely on the phase I data and preliminary CD4 count data from the randomized trials. This approval gave physicians another tool to fight HIV infection, and, as described below, it became possible for physicians to consider combination therapy.

1.7 Impact of Anti-HIV Drugs

1.7.1 *Highly Active Antiretroviral Therapy (HAART)*

Based in part on the experience learned in developing combination chemotherapy for lymphoma and other tumors, our group felt it likely that the treatment of HIV would ultimately require drug combinations and started exploring this in the laboratory and clinic from the outset (Mitsuya et al. 1987b; Nguyen et al. 1993; Surbone et al. 1988; Yarchoan et al. 1988). On an extension of the phase I trial of ddI, we switched patients to a combination of AZT and ddI and found that some with advanced HIV infection or AIDS could survive for 5 years or longer on this combination (Nguyen et al. 1995). In addition, several randomized trials showed that AZT or other dideoxynucleosides used in combination were better than AZT alone and that simultaneous use of these drugs was significantly better than their alternating use (Collier et al. 1993; Hammer et al. 1996; Yarchoan et al. 1994). During this period, work progressed on other HIV targets, and with the development of first protease inhibitors (Kempf et al. 1990; McQuade et al. 1990; Meek et al. 1990;

Miller et al. 1989; Roberts et al. 1990), it was possible to test combinations of three drugs including two dideoxynucleosides and a protease inhibitor (Hammer et al. 1997). The testing of such regimens was greatly aided by development of methodologies to measure the viral load in plasma and to assess HIV resistance (Hirsch et al. 1998; Mellors et al. 1996; Perelson et al. 1996). Certain regimens utilizing three or more drugs, called highly active antiretroviral therapy (HAART), were found to suppress HIV replication to undetectable levels in many patients, so that the development of HIV drug resistance was halted or substantially slowed (Hammer et al. 1997). This approach resulted in substantial immunologic restoration, clinical improvement, and prolonged survival so that AIDS was converted from a rapidly fatal disease to a manageable chronic illness. When HAART regimens were developed, much of the focus was on the contribution of the protease inhibitors. However, it should be remembered that HAART regimens generally included two NRTI such as zidovudine and didanosine and that three-drug regimens utilizing three NRTI were subsequently also found to have excellent activity (Bonjoch et al. 2005), although perhaps not as lasting as regimens utilizing two classes of drugs. It is also noteworthy that despite much attention given to protease and integrase inhibitors, NRTIs have remained the backbone of HIV therapy 25 years after their introduction into the clinic. Contributing to the long-term utility of NRTI are their properties of being relatively inexpensive to synthesize, being co-formulated easily with other drugs, and having relatively minor pharmacokinetic interactions. Also, in many cases, resistance to these drugs developed more slowly than to drugs in other classes.

1.8 Prevention of HIV Transmission

Another substantial health benefit of AZT was in preventing mother-to-child and other HIV transmission. A short course of antepartum AZT, intrapartum AZT, and 6-week administration of AZT to the newborn child reduced perinatal transmission by approximately two-thirds in an initial trial (Connor et al. 1994), and adopting this and related anti-HIV therapy around the time of birth has all but eradicated peripartum transmission of HIV. In addition, recent studies have showed that administration of NRTI and other anti-HIV drugs can substantially reduce HIV transmission in discordant couples (Celum and Baeten 2012; Grant et al. 2010), and such pre-exposure prophylaxis (PrEP) now holds great promise as a strategy to reduce the spread of HIV (Cohen 2011).

1.9 Conclusions

When HIV was identified as the causative agent of AIDS, there was hope that this would lead to an effective vaccine in short order, and the development of effective antiviral therapy seemed far less promising. In the April 24, 1984, New York Times

report of the press conference given by the Secretary of Health and Human Services to announce the discovery of the cause of AIDS, it was stated that “Finding the cause of AIDS will not necessarily lead to any treatment of the disease soon.... But the finding led the American researchers to express the hope that a vaccine would be developed and ready for testing in ‘about 2 years’” (Altman 1984). At that time, patients with HIV infection almost always progressed to AIDS within 10 years or so, and survival of AIDS patients was measured in months. Now, 28 years later, there is no HIV vaccine available, and the most promising lead in this area is a recent clinical trial showing suggestive evidence of a statistically significant but slight protective effect with a prototype vaccine (Rerks-Ngarm et al. 2009). By contrast, effective anti-HIV drugs were rapidly developed and the first approved for use within 3 years; with current combination regimens, the life expectancy of HIV-infected individuals treated with antiretroviral therapy is better than that of individuals with type I diabetes and in some groups is approaching that of the general population (Lewden et al. 2012; Lohse et al. 2007). Moreover, antiretroviral therapy has proven to be effective in preventing mother-to-child transmission and even in preventing transmission of HIV between sexual partners.

During the development of AZT and other dideoxynucleosides, it became apparent early on that these drugs had substantial limitations; at tolerable concentrations, HIV replication was only partially inhibited *in vitro*, and patients receiving these drugs had only partial and transient clinical improvement, often accompanied by substantial toxicity. We never claimed that these drugs, by themselves, would be the answer to AIDS. Nonetheless, it can be argued that their development led the way to the development of other drugs and that regimens using these drugs have dramatically changed the face of AIDS.

Looking back, the intramural program of the NCI in the 1980s provided an outstanding environment that facilitated the rapid development of early AIDS drugs (Broder 2010a, b). In part because of the “Special Virus-Cancer Program,” the NCI had considerable expertise in retroviruses, and soon after the Gallo group developed the ability to isolate and grow HIV, we were able to rapidly transfer this technology to our laboratory. Our group also had considerable immunologic expertise from our previous studies of immunodeficiency disease. The NCI as a whole had substantial expertise in nucleoside chemistry and had an established drug development program that we could tap into for help in animal toxicology, formulation, and regulatory support. Working in an oncologic environment, we were willing to accept and to be attuned for small advances. Also, during this period, we were able to do much of our work as a small, isolated “SWAT team” and were not slowed by a requirement to go through multiple committees for each step of the process. Indeed, because our group encompassed both a laboratory and clinical effort, we could bring drugs to the clinic without a formal hand-off from one group to the other. We should note that the FDA at the time recognized the urgency to develop drugs for AIDS and expeditiously reviewed the applications and protocols for these trials.

During this period of early AIDS drug development, there was considerable controversy that NCI resources were being used in what was viewed as a distraction from the main mission of the NCI – cancer research. However, it is worth noting that

these drugs, along with other anti-HIV drugs, dramatically reduced the incidence of AIDS malignancies, especially those associated with low CD4 counts such as Kaposi's sarcoma or primary central nervous system lymphoma (Engels et al. 2006; Herida et al. 2003; Jones et al. 1998). Thus, this work has in fact proven to be extremely important in lowering the morbidity and mortality from AIDS-defining cancers – a goal that is very much within the mission of the NCI.

The NCI continues to contribute to the development of anti-HIV therapy, and Mitsuya and colleagues have more recently developed a fourth approved anti-HIV drug, the protease inhibitor darunavir (Koh et al. 2003). This drug has been licensed royalty free to the Medicines Patent Pool, a UNITAID/WHO initiative that aims to provide new and effective drugs to developing countries. This is a response by the United States Government and the NCI to critical the need for such drugs in resource-limited areas. Along those same lines, in 2003, the United States Government embarked on a program to make antiretroviral therapy available in much of the developing world through the President's Emergency Plan for AIDS Relief (PEPFAR) (Broder 2010a; Walensky and Kuritzkes 2010). Billions of dollars have been committed to PEPFAR, and along with public health measures to reduce transmission, we are finally starting to see a reversal of the AIDS epidemic in many regions of the developing world. It is estimated that more than 1.2 million deaths have been avoided in 5 years by antiretroviral drugs provided by PEPFAR in South Africa alone (Walensky and Kuritzkes 2010). It should also be noted that with advances in the cost-efficient manufacture of antiretroviral drugs and their being made available royalty free, antiretroviral therapy can now be administered in the developing world for less than \$350 per person per year (Goosby 2012). This accomplishment is noteworthy in light of the concern raised during the initial development of AZT and related drugs that they were too expensive to be used in the developing world.

Conflict of Interest RY, HM, and SB are coinventors on one or more US and/or foreign patents involving dideoxycytidine (zalcitabine), dideoxyadenosine, dideoxyinosine (didanosine), and zidovudine; HM is also coinventor on patents involving darunavir. These inventions were all made as full-time employees of the United States Government under 45 Code of Federal Regulations Part 7. All rights, title, and interest to these patents are assigned to the U.S. Department of Health and Human Services. The government conveys a portion of the royalties it receives to its employee-inventors under the Federal Technology Transfer Act of 1986 (P.L. 99-502).

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GRL-0519, a Novel Oxatricyclic Ligand-Containing Nonpeptidic HIV-1 Protease Inhibitor (PI), Potently Suppresses Replication of a Wide Spectrum of Multi-PI-Resistant HIV-1 Variants *In Vitro*

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We report that GRL-0519, a novel nonpeptidic human immunodeficiency virus type 1 (HIV-1) protease inhibitor (PI) containing *tris*-tetrahydrofuranylurethane (*tris*-THF) and a sulfonamide isostere, is highly potent against laboratory HIV-1 strains and primary clinical isolates (50% effective concentration [EC₅₀], 0.0005 to 0.0007 μM) with minimal cytotoxicity (50% cytotoxic concentration [CC₅₀], 44.6 μM). GRL-0519 blocked the infectivity and replication of HIV-1_{NL4-3} variants selected by up to a 5 μM concentration of ritonavir, lopinavir, or atazanavir (EC₅₀, 0.0028 to 0.0033 μM). GRL-0519 was also potent against multi-PI-resistant clinical HIV-1 variants isolated from patients who no longer responded to existing antiviral regimens after long-term antiretroviral therapy, highly darunavir (DRV)-resistant variants, and HIV-2_{ROD}. The development of resistance against GRL-0519 was substantially delayed compared to other PIs, including amprenavir (APV) and DRV. The effects of nonspecific binding of human serum proteins on GRL-0519's antiviral activity were insignificant. Our analysis of the crystal structures of GRL-0519 (3OK9) and DRV (2IEN) with protease suggested that the *tris*-THF moiety, compared to the *bis*-THF moiety present in DRV, has greater water-mediated polar interactions with key active-site residues of protease and that the *tris*-THF moiety and paramethoxy group effectively fill the S2 and S2' binding pockets, respectively, of the protease. The present data demonstrate that GRL-0519 has highly favorable features as a potential therapeutic agent for treating patients infected with wild-type and/or multi-PI-resistant variants and that the *tris*-THF moiety is critical for strong binding of GRL-0519 to the HIV protease substrate binding site and appears to be responsible for its favorable antiretroviral characteristics.

Combination antiretroviral therapy (cART) has had a major impact on the AIDS epidemic in industrially advanced nations. Recent analyses have revealed that mortality rates for human immunodeficiency virus type 1 (HIV-1)-infected persons have become close to those of the general population (1–4). However, eradication of HIV-1 does not appear to be currently possible, in part due to the viral reservoirs remaining in blood and infected tissues. Moreover, we have encountered a number of challenges in bringing the optimal benefits of the currently available therapeutics for AIDS and HIV-1 infection to individuals receiving cART (5–7). They include (i) drug-related toxicities, (ii) inability to fully restore normal immunologic functions once individuals developed AIDS, (iii) development of various cancers as a consequence of survival prolongation, (iv) flaring up of inflammation in individuals receiving cART or immune reconstruction syndrome (IRS), and (v) increased cost of antiviral therapy. Such limitations and flaws of cART are exacerbated by the development of drug-resistant HIV-1 variants (8–12), although the recent first-line cART with boosted protease inhibitor (PI)-based regimens has made the development of HIV-1 resistance less likely over an extended period of time (13).

Successful antiviral drugs, in theory, produce their virus-specific effects by interacting with viral receptors, virus-encoded enzymes, viral structural components, viral genes, or their transcripts without disturbing cellular metabolism or function. However, at present, no antiretroviral drugs or agents are likely to be completely specific for HIV-1 or to be devoid of toxicity or side effects in the therapy of AIDS. This is a critical issue, because

patients with AIDS and its related diseases will have to receive antiretroviral therapy for a long time, perhaps for the rest of their lives. Thus, the identification of new classes of antiretroviral drugs that have a unique mechanism(s) of action and produce no or minimal side effects remains an important therapeutic objective.

We have been focusing on the design and synthesis of nonpeptidyl PIs that are potent against HIV-1 variants resistant to the currently approved PIs. One such anti-HIV-1 agent, darunavir (DRV) (Fig. 1), containing a structure-based designed privileged nonpeptidic P2 ligand, 3(*R*),3a(*S*),6a(*R*)-*bis*-tetrahydrofuranylurethane (*bis*-THF) (14–16), has been approved as a first-line therapeutic agent for the treatment of individuals who are infected with HIV-1. In the present work, we examined and characterized the nonpeptidic HIV-1 protease inhibitors GRL-0519 (17) and its stereoisomer, GRL-0529, both of which contain the *tris*-THF moiety and a sulfonamide isostere. We found that GRL-0519 exerts highly potent activity against a wide spectrum of laboratory HIV-1

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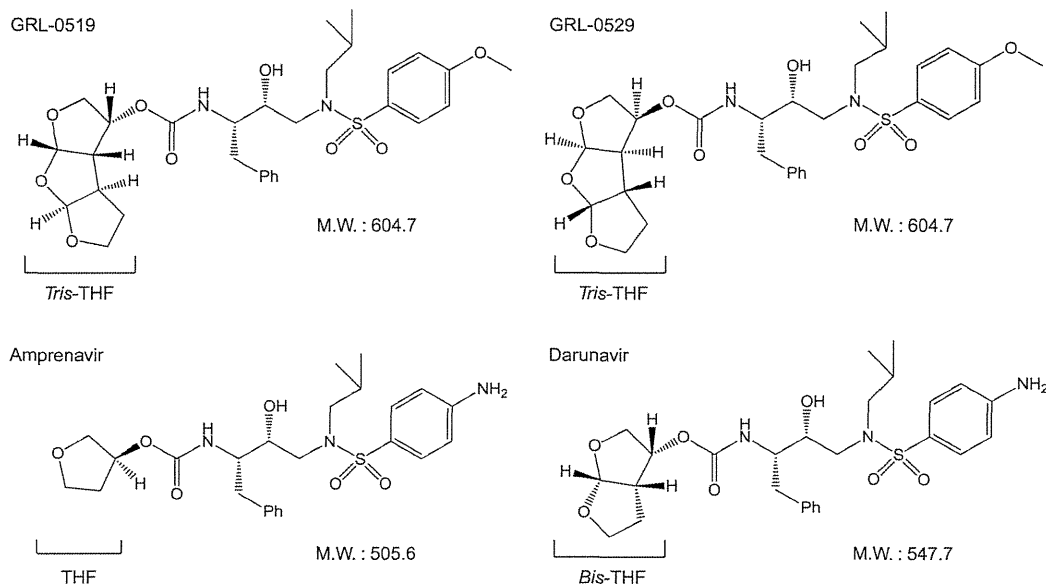


FIG 1 Structures of GRL-0519, GRL-0529, amprenavir, and darunavir. M.W., molecular weight.

strains and primary clinical isolates, including multi-PI-resistant variants with minimal cytotoxicity. In addition, GRL-0519 was active against HIV-2_{ROD}, as well as HIV-1 isolates examined. We also selected HIV-1 variants with GRL-0519 by propagating a laboratory wild-type HIV-1_{NL4-3} in MT-4 cells in the presence of increasing concentrations of GRL-0519 and determined the amino acid substitutions that emerged under the pressure of GRL-0519 in the protease-encoding region. In addition, we evaluated the effects of nonspecific binding of physiological human serum proteins on GRL-0519's anti-HIV-1 activity. We further analyzed the previously published crystal structure of GRL-0519 with protease (Protein Data Bank [PDB] ID, 3OK9) to gain a better understanding of the present antiviral data. The crystal structure analyses indicated that GRL-0519 has strong polar interactions with key residues in the active site of the protease. GRL-0519 also has several water-mediated polar interactions and tight van der Waals interactions with protease residues, suggesting that GRL-0519 binds very tightly in the active site of the protease.

MATERIALS AND METHODS

Cells and viruses. MT-2 and MT-4 cells were grown in RPMI 1640-based culture medium supplemented with 10% fetal calf serum (FCS) (JRH Biosciences, Lenexa, MD), 50 units/ml penicillin, and 100 μ g/ml kanamycin. The following HIV strains were employed for the drug susceptibility assay (see below): HIV-1_{LAI}, HIV-1_{NL4-3}, HIV-2_{ROD}, HIV-1_{ERS104pre} (18), clinical HIV-1 strains isolated from drug-naïve patients with AIDS, and six HIV-1 clinical strains that were originally isolated from patients with AIDS who had received 9 to 11 anti-HIV-1 drugs over the past 32 to 83 months and that were genotypically and phenotypically characterized as multi-PI-resistant HIV-1 variants (19, 20). All primary HIV-1 strains were passaged once or twice in 3-day-old phytohemagglutinin-activated peripheral blood mononuclear cells (PHA-PBM), and the virus-containing culture supernatants were stored at -80°C until they were used as sources of infectious virions.

Antiviral agents and human serum proteins. Roche Products Ltd. (Welwyn Garden City, United Kingdom) and Abbott Laboratories (Abbott Park, IL) kindly provided saquinavir (SQV) and ritonavir (RTV), respectively. Amprenavir (APV) was a courtesy gift from GlaxoSmith-

Kline, Research Triangle Park, NC. Lopinavir (LPV) was kindly provided by Japan Energy Inc., Tokyo, Japan. Atazanavir (ATV) was a contribution from Bristol Myers Squibb (New York, NY). Darunavir (DRV) was synthesized as previously described (21). Human serum albumin (HSA) and α 1-acid glycoprotein (AAG) were purchased from Sigma-Aldrich (St. Louis, MO).

Drug susceptibility assay. The susceptibility of HIV-1_{LAI} or HIV-2_{ROD} to various drugs was determined as previously described with minor modifications. Briefly, MT-2 cells (10^4 /ml) were exposed to 100 50% tissue culture infectious doses (TCID₅₀) of HIV-1_{LAI} or HIV-2_{ROD} in the presence or absence of various concentrations of drugs in 96-well microculture plates and incubated at 37°C for 7 days. After incubation, 100 μ l of the medium was removed from each well and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (10 μ l, 7.5 mg/ml in phosphate-buffered saline) was added to each well in the plate, followed by incubation at 37°C for 2 h. After incubation to dissolve the formazan crystals, 100 μ l of acidified isopropanol containing 4% (vol/vol) Triton X-100 was added to each well, and the optical density was measured in a kinetic microplate reader (Vmax; Molecular Devices, Sunnyvale, CA). All assays were performed in duplicate or triplicate. In some experiments, MT-2 cells were chosen as target cells in the MTT assay, since these cells undergo greater HIV-1-elicited cytopathic effects than MT-4 cells. To determine the sensitivity of primary HIV-1 isolates to drugs, PHA-PBM (10^6 /ml) were exposed to 50 TCID₅₀ of each primary HIV-1 isolate and cultured in the presence or absence of various concentrations of drugs in 10-fold serial dilutions in 96-well microculture plates. In determining the drug susceptibilities of certain laboratory HIV-1 strains, MT-4 cells were employed as target cells as previously described, with minor modifications. In brief, MT-4 cells (10^3 /ml) were exposed to 100 TCID₅₀ of drug-resistant HIV-1 strains in the presence or absence of various concentrations of drugs and incubated at 37°C . On day 7 of culture, the supernatants were harvested and the amounts of p24 (capsid [CA]) Gag protein were determined by using a fully automated chemiluminescent enzyme immunoassay system (Lumipulse F; Fujirebio Inc., Tokyo, Japan) (22, 23). Drug concentrations that suppressed the production of p24 Gag protein by 50% (50% effective concentration [EC₅₀]) were determined by comparison with the p24 production level in a drug-free control cell culture. All assays were performed in duplicate or triplicate. PHA-PBM were derived from a single donor in each independent experiment. Thus, to obtain the data,

three different healthy donors were recruited. For determining the anti-retroviral activity and cytotoxicity of a drug, we used the same cells and cultured them for the same 7 days. The MTT assay was employed for HIV-1_{LAI} and HIV-2_{ROD}, while the p24 assay was employed for clinical HIV-1 isolates and drug-resistant HIV-1 strains.

Creation of PI-resistant HIV-1 variants *in vitro*. MT-4 cells (10^5 /ml) were exposed to HIV-1_{NL4-3} (500 TCID₅₀) and cultured in the presence of various PIs at an initial concentration equal to its EC₅₀. Viral replication was monitored by the determination of the amount of p24 Gag produced by MT-4 cells. The culture supernatants were harvested on day 7 and were used to infect fresh MT-4 cells for the next round of culture in the presence of increasing concentrations of each drug. When the virus began to propagate in the presence of the drug, the drug concentration was increased generally 2- to 3-fold. Proviral DNA samples obtained from the lysates of infected cells were subjected to nucleotide sequencing. This drug selection procedure was carried out until the drug concentration reached 5 μ M, as previously described (24–26). In the experiments for selecting drug-resistant variants, MT-4 cells were also exploited as target cells, since HIV-1 in general replicates at higher levels in MT-4 cells than in MT-2 cells, as described above.

Determination of nucleotide sequences. Molecular cloning and determination of the nucleotide sequences of HIV-1 strains passaged in the presence of anti-HIV-1 agents were performed as previously described (24). In brief, high-molecular-weight DNA was extracted from HIV-1-infected MT-4 cells by using the InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) and was subjected to molecular cloning, followed by sequence determination. The primers used for the first round of PCR with the entire Gag- and protease-encoding regions of the HIV-1 genome were LTR F1 (5'-GAT GCT ACA TAT AAG CAG CTG C-3') and PR12 (5'-CTC GTG ACA AAT TTC TAC TAA TGC-3'). The first-round PCR mixture consisted of 1 μ l of proviral DNA solution, 10 μ l of Premix *Taq* (Ex *Taq* version; TaKaRa Bio Inc., Otsu, Japan), and 10 pmol of each of the first PCR primers in a total volume of 20 μ l. The PCR conditions used were an initial 3 min at 95°C, followed by 35 cycles of 40 s at 95°C, 20 s at 55°C, and 2 min at 72°C, with a final 10 min of extension at 72°C. The first-round PCR products (1 μ l) were used directly in the second round of PCR with primers LTR F2 (5'-GAG ACT CTG GTA ACT AGA GAT C-3') and KSM2.1 (5'-CCA TCC CGG GCT TTA ATT TTA CTG GTA C-3') under the following PCR conditions: an initial 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 20 s at 55°C, and 2 min at 72°C, with a final 10 min of extension at 72°C (a stick diagram of HIV-1 genome PCR amplification for sequence analysis is shown in Fig. S1 in the supplemental material). The second-round PCR products were purified with spin columns (MicroSpin S-400 HR columns; Amersham Biosciences Corp., Piscataway, NJ), cloned directly, and subjected to sequencing with a model 3130 automated DNA sequencer (Applied Biosystems, Foster City, CA).

Determination of replication kinetics of GRL-0519-resistant HIV-1_{NL4-3} variants and wild-type HIV-1_{NL4-3}. The GRL-0519-resistant variant at passage 37 was propagated in fresh MT-4 cells without GRL-0519 for 7 days, and aliquoted HIV-1₅₁₉^R_{P37} viral stocks were stored at -80°C until use. MT-4 cells (3.2×10^5) were exposed to the HIV-1₅₁₉^R_{P37} or wild-type HIV-1_{NL4-3} preparation containing 10 ng/ml p24 in 6-well culture plates for 3 h, and the newly infected MT-4 cells were washed with fresh medium and divided into 4 fractions, each cultured with or without GRL-0519 (final concentration of MT-4 cells, 10^4 /ml; drug concentrations, 0, 0.005, 0.01, and 0.015 μ M). The amounts of p24 were measured every 2 days for up to 7 days.

Generation of recombinant HIV-1 clones. To generate HIV-1 clones carrying the desired amino acid substitutions, site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), and the amino acid substitution-containing genomic fragments were introduced into pHIV-1_{NL4-3Sma}. Determination of the nucleotide sequences of the plasmids confirmed that each clone had the desired amino acid substitution but no unintended amino acid substitutions. Each recombinant plasmid was transfected into COS7 cells with

TABLE 1 Antiviral activities of GRL-0519 and -0529 against HIV-1_{LAI} or HIV-2_{ROD}

Compound	EC ₅₀ (μ M) ^a		CC ₅₀ (μ M) (\pm SD)	Selectivity index ^b
	HIV-1 _{LAI}	HIV-2 _{ROD}		
GRL-0519	0.0007 \pm 0.0005	0.0004 \pm 0.0002	44.6 \pm 3.5	63,714
GRL-0529	0.33 \pm 0.04	0.40 \pm 0.06	38.7 \pm 3.7	118
SQV	0.026 \pm 0.006	0.003 \pm 0.001	19.8 \pm 2.9	773
APV	0.033 \pm 0.002	0.37 \pm 0.11	84.7 \pm 5.4	2,590
ATV	0.0048 \pm 0.0001	0.0077 \pm 0.0006	27.6 \pm 2.7	5,520
DRV	0.0042 \pm 0.0006	0.0088 \pm 0.0004	152.7 \pm 10.1	36,357

^a MT-2 cells (10^4 /ml) were exposed to 100 TCID₅₀ of HIV-1_{LAI} or HIV-2_{ROD} and cultured in the presence of various concentrations of each PI, and the EC₅₀ values were determined using the MTT assay. All assays were conducted in duplicate, and the data shown represent mean value (\pm 1 standard deviation [SD]) derived from the results of three independent experiments.

^b Each selectivity index denotes a ratio of CC₅₀ to EC₅₀ against HIV-1_{LAI}.

Lipofectamine LTX transfection reagent (Invitrogen, Carlsbad, CA), and the infectious virions thus made were harvested for 72 h after transfection and stored at -80°C until use.

Structural analysis of interactions of GRL-0519 and DRV with protease. The crystal structures of HIV-1 protease complexed with GRL-0519 or DRV were obtained from the protein data bank (PDB ID, 3OK9 and 2IEN, respectively). The inhibitor conformation with the higher occupancy in the crystal structure was considered for analysis. Bond orders were properly assigned to the inhibitor molecules. Hydrogens were added to all the heavy atoms, and their positions were optimized in an OPLS2005 force field (27) with constraints on heavy atom positions. A cutoff distance of 3.0 Å between a polar hydrogen and oxygen or nitrogen was used to determine the presence of hydrogen bonds. The structures were analyzed using Maestro version 9.3 (Schrödinger, LLC, New York, NY, 2012).

RESULTS

Antiviral activities of GRL-0519 and -0529 against HIV-1_{LAI} and HIV-2_{ROD} and their cytotoxicities. We first examined the antiviral potencies of GRL-0519 and -0529 against a variety of HIV-1 isolates. GRL-0529 showed only moderate anti-HIV-1 activity against a laboratory wild-type HIV-1 strain, HIV-1_{LAI}, and an HIV-2 strain, HIV-2_{ROD}, with EC₅₀s of 0.33 and 0.40 μ M, respectively (Table 1). Conversely, GRL-0519 was extremely potent against HIV-1_{LAI}, with an EC₅₀ of 0.0007 μ M compared to other clinically available Food and Drug Administration (FDA)-approved PIs examined, including DRV (Table 1), as assessed with the MTT assay using MT-2 target cells, while its cytotoxicity was evident only at high concentrations (50% cytotoxic concentration [CC₅₀], 44.6 μ M) and the selectivity index proved to be highly favorable at 63,714 (Table 1). GRL-0519 was also very potent against HIV-2_{ROD}, with an EC₅₀ of 0.0004 μ M (Table 1).

GRL-0519 is potent against various PI-selected laboratory HIV-1 variants. We also examined GRL-0519 against an array of HIV-1_{NL4-3} variants, which had been selected by propagating HIV-1_{NL4-3} in the presence of increasing concentrations (up to 5 μ M) of each of 4 FDA-approved PIs (RTV, APV, ATV, and LPV) in MT-4 cells (24). Such variants had acquired various PI resistance-associated amino acid substitutions in the protease-encoding region of the viral genome (Table 2, note a). Each variant was highly resistant to the PI by which the variant was selected and showed significant resistance, with an EC₅₀ of >1 μ M. GRL-0519 was highly active against all the variants (except HIV-1_{APV}^R_{5 μ M}), with EC₅₀s of 2.8 to 3.3 nM (differences were 6- to 7-fold greater compared to those against HIV-1_{NL4-3}) (Table 2).

TABLE 2 Antiviral activities of GRL-0519 and -0529 against laboratory PI-resistant HIV-1 variants

Virus ^a	EC ₅₀ (nM) ^b						
	GRL-0519	GRL-0529	APV	ATV	LPV	TPV	DRV
HIV-1 _{NL4-3}	0.5 ± 0.1	356.3 ± 33.8	24.9 ± 0.1	4.2 ± 1.1	37.8 ± 4.5	362.9 ± 104.4	3.9 ± 0.6
HIV-1 _{RTV} ^R _{5μM}	2.8 ± 0.4 (6)	504.3 ± 26.6 (1)	532.9 ± 25.9 (21)	36.4 ± 3.0 (9)	501.2 ± 44.9 (13)	402.1 ± 56.1 (1)	29.1 ± 2.1 (7)
HIV-1 _{APV} ^R _{5μM}	38.0 ± 0.9 (76)	>1,000 (>3)	>1,000 (>40)	371.0 ± 7.8 (88)	>1,000 (>26)	>1,000 (>3)	368.5 ± 32.4 (94)
HIV-1 _{ATV} ^R _{5μM}	3.3 ± 1.6 (7)	376.9 ± 164.8 (1)	390.0 ± 11.1 (16)	>1,000 (>238)	>1,000 (>26)	>1,000 (>3)	28.1 ± 6.4 (7)
HIV-1 _{LPV} ^R _{5μM}	3.2 ± 0.4 (7)	>1,000 (>3)	420.1 ± 61.9 (17)	35.1 ± 4.7 (8)	>1,000 (>26)	>1,000 (>3)	32.9 ± 1.3 (8)

^a The amino acid substitutions identified in the protease-encoding region compared to the wild-type HIV-1_{NL4-3} include the following: M46I, V82F, and I84V in HIV-1_{RTV}^R_{5μM}; L10F, V32I, M46I, I54 M, A71V, and I84V in HIV-1_{APV}^R_{5μM}; L23I, E34Q, K43I, M46I, I50L, G51A, L63P, A71V, V82A, and T91A in HIV-1_{ATV}^R_{5μM}; and L10F, M46I, I54V, and V82A in HIV-1_{LPV}^R_{5μM}.

^b The EC₅₀ values were determined by using MT-4 cells as target cells. MT-4 cells (10⁵/ml) were exposed to 100 TCID₅₀ of each HIV-1 strain, and the inhibition of p24 Gag protein production by each drug was used as an endpoint. The numbers in parentheses represent the fold changes of EC₅₀s for each isolate compared to the EC₅₀s for HIV-1_{NL4-3}. All assays were conducted in duplicate or triplicate, and the data shown represent mean values (± 1 SD) derived from the results of two or three independent experiments.

GRL-0519 exerts potent activity against highly PI-resistant clinical HIV-1 isolates. In our previous work, we isolated highly multi-PI-resistant primary HIV-1 strains, HIV-1_{MDR/B}, HIV-1_{MDR/C}, HIV-1_{MDR/G}, HIV-1_{MDR/TM}, HIV-1_{MDR/MM}, and HIV-1_{MDR/JSL}, from patients with AIDS who had failed then-existing anti-HIV regimens after receiving 9 to 11 anti-HIV-1 drugs over 32 to 83 months (19, 20). These primary strains contained 9 to 14 amino acid substitutions in the protease-encoding region, which have reportedly been associated with HIV-1 resistance against various PIs (Table 3, note a). The potency of APV, ATV, and LPV against such clinical multidrug-resistant HIV-1 strains was significantly compromised, as examined in PHA-PBM as target cells using p24 production inhibition as an endpoint (Table 3). However, GRL-0519 exerted quite potent antiviral activity, and its EC₅₀s against those clinical variants were quite low: 0.8 to 3.4 nM (Table 3). The antiviral activity of GRL-0519 proved to be most

potent against the six multidrug-resistant clinical HIV-1 variants examined compared to the four FDA-approved PIs (APV, ATV, LPV, and DRV). We also examined the antiviral activity of GRL-0519 against highly DRV-resistant variants (28). These variants were created using the mixture of 8 highly multi-PI-resistant clinical isolates as a starting HIV-1 source and selected with increasing concentrations of DRV. GRL-0519 maintained its activity against highly DRV-resistant MDR mixtures (EC₅₀, 5.6 to 30.0 nM), being more potent than DRV by 7.8- to 8.5-fold (Table 3). Overall, GRL-0519 exerted stronger antiviral activity against various wild-type HIV-1 strains, drug-resistant variants, and HIV-2 strains than DRV by 5- to 22-fold. Furthermore, GRL-0519 was more potent than APV by 118- to 925-fold against HIV-2_{ROD} and drug-resistant variants (Tables 1 to 3).

Effects of human serum proteins on the antiretroviral activity of GRL-0519. The binding of human serum proteins to a

TABLE 3 Antiviral activities of GRL-0519 and -0529 against multidrug-resistant clinical isolates and highly DRV-resistant MDR mixtures in PHA-PBM

Virus ^a	EC ₅₀ (nM) ^b					
	GRL-0519	GRL-0529	APV	ATV	LPV	DRV
HIV-1 _{WT/ERS104pre}	0.6 ± 0.2	347.4 ± 27.3	33.8 ± 5.1	2.7 ± 0.6	31.4 ± 4.2	3.9 ± 0.6
HIV-1 _{MDR/B} (X4)	3.4 ± 0.5 (6)	611.8 ± 72.8 (2)	459.4 ± 99.2 (14)	469.7 ± 7.4 (174)	> 1,000 (>32)	27.8 ± 5.9 (7)
HIV-1 _{MDR/C} (X4)	0.8 ± 0.2 (1)	514.4 ± 130.6 (1)	346.1 ± 55.2 (10)	38.8 ± 2.8 (14)	436.5 ± 3.5 (14)	10.3 ± 2.4 (3)
HIV-1 _{MDR/G} (X4)	2.6 ± 1.3 (4)	655.8 ± 292.9 (2)	462.6 ± 64.5 (14)	19.4 ± 7.5 (7)	181.3 ± 23.0 (6)	27.8 ± 5.2 (7)
HIV-1 _{MDR/TM} (X4)	2.1 ± 0.3 (4)	530.0 ± 74.7 (2)	476.4 ± 8.1 (14)	74.5 ± 2.6 (28)	422.9 ± 82.0 (13)	30.0 ± 1.0 (8)
HIV-1 _{MDR/MM} (R5)	2.5 ± 0.5 (4)	787.4 ± 251.4 (2)	338.9 ± 15.5 (10)	204.8 ± 23.5 (76)	622.5 ± 82.0 (20)	13.3 ± 6.2 (3)
HIV-1 _{MDR/JSL} (R5)	2.5 ± 0.2 (4)	> 1,000 (>3)	436.3 ± 90.4 (13)	211.3 ± 98.6 (78)	> 1,000 (>32)	22.1 ± 9.3 (6)
HIV-1 _{DRV} ^R _{10P}	5.6 ± 0.4 (9)	> 1,000 (>3)	> 1,000 (>32)	322.9 ± 10.4 (77)	> 1,000 (>32)	43.4 ± 13.4 (11)
HIV-1 _{DRV} ^R _{20P}	30.0 ± 9.8 (50)	> 1,000 (>3)	> 1,000 (>32)	> 1,000 (>370)	> 1,000 (>32)	255.2 ± 4.0 (64)

^a The amino acid substitutions identified in the protease-encoding region compared to the consensus type B sequence cited from the Los Alamos database include the following: L63P in HIV-1_{ERS104pre}; L10I, K14R, L33I, M36I, M46I, F53I, K55R, I62V, L63P, A71V, G73S, V82A, L90 M, and I93L in HIV-1_{MDR/B}; L10I, I15V, K20R, L24I, M36I, M46L, I54V, I62V, L63P, K70Q, V82A, and L89 M in HIV-1_{MDR/C}; L10I, V11I, T12E, I15V, L19I, R41K, M46L, L63P, A71T, V82A, and L90 M in HIV-1_{MDR/G}; L10I, K14R, R41K, M46L, I54V, L63P, A71V, V82A, L90 M, and I93L in HIV-1_{MDR/TM}; L10I, K43T, M46L, I54V, L63P, A71V, V82A, L90 M, and Q92K in HIV-1_{MDR/MM}; L10I, L24I, I33F, E35D, M36I, N37S, M46L, I54V, R57K, I62V, L63P, A71V, G73S, and V82A in HIV-1_{MDR/JSL}; L10I, I15V, K20R, L24I, V32I, M36I, M46L, I54V, I62V, L63P, K70Q, V82A, and L88 M in HIV-1_{DRV}^R_{10P}; and L10I, I15V, K20R, L24I, V32I, M36I, M46L, L63P, A71T, V82A, and L88 M in HIV-1_{DRV}^R_{20P}. HIV-1_{ERS104pre} served as a source of wild-type HIV-1. DRV-resistant HIV-1 variants (HIV-1_{DRV}^R_{10P} and HIV-1_{DRV}^R_{20P}) were selected *in vitro* by propagating a mixture of eight HIV-1_{MDR} isolates in the presence of increasing concentrations of DRV in MT-4 cells. Six of the eight isolates were the same as those described above. Amino acid substitutions identified in proteases of the other two isolates compared to the consensus type B sequence cited from the Los Alamos database include the following: L10I, I15V, E35D, N37E, K45R, I54V, L63P, A71V, V82T, L90 M, I93L, and C95F in HIV-1_{MDR/A}; L10R, N37D, M46I, I62V, L63P, A71V, G73S, V74I, V82T, L90 M, and I93L in HIV-1_{MDR/SS}.

^b The EC₅₀ values were determined by using PHA-PBM as target cells, and the inhibition of p24 Gag protein production by each drug was used as an endpoint. The numbers in parentheses represent the fold changes of EC₅₀s for each isolate compared to the EC₅₀s for HIV-1_{ERS104pre}. All assays were conducted in duplicate or triplicate, and the data shown represent mean values (± 1 SD) derived from the results of three independent experiments. PHA-PBM were derived from a single donor in each independent experiment.

TABLE 4 Nonspecific binding effects of human serum proteins on GRL-0519's antiviral activity

Compound	EC ₅₀ (nM) ^a		
	None	HSA	AAG
GRL-0519	0.6 ± 0.2	1.9 ± 1.1 (3)	2.4 ± 0.3 (4)
GRL-0529	347.4 ± 27.3	351.5 ± 22.7 (2)	649.0 ± 48.9 (2)
APV	31.5 ± 5.5	30.5 ± 2.6 (1)	286.3 ± 14.8 (9)
ATV	2.7 ± 0.6	14.6 ± 1.2 (5)	32.7 ± 15.9 (12)
LPV	31.4 ± 4.2	32.5 ± 2.7 (1)	232.1 ± 15.2 (7)
DRV	3.9 ± 1.0	7.4 ± 1.2 (2)	33.8 ± 3.5 (9)

^a HSA (40 mg/ml) or AAG (10 μM) was used to evaluate the binding effects of human serum proteins on GRL-0519 and -0529 antiviral activities. The EC₅₀ values against HIV-1_{ERS104pre} with or without HSA or AAG were determined by p24 assay using PHA-PBM as target cells, and the inhibition of p24 Gag production by each drug was used as an endpoint. The numbers in parentheses represent the fold changes of the EC₅₀ values compared to the values without HSA or AAG. The data shown represent mean values derived from the results of two or three independent experiments. PBM were derived from a single donor in each independent experiment.

drug is an important determinant of its pharmacological activity *in vivo*, because overly tight binding may result in reducing interactions between the drug and its target (29). We thus determined the effects of the binding of HSA and AAG on GRL-0519's antiretroviral activity *in vitro*. Physiologically normal concentrations of HSA (40 mg/ml) and AAG (10 μM) were used to evaluate their binding effects on GRL-0519's activity against a wild-type clinical isolate, HIV-1_{ERS104pre}. All four FDA-approved drugs substantially maintained their activity in the presence of HSA, with reduction of activity by up to 5-fold relative to the activity in the absence of additional HSA (Table 4). The activities of those PIs were reduced in the presence of AAG by 7- to 12-fold (Table 4). However, the binding effects of both HSA and AAG on GRL-0519's activity were insignificant, only a 3- to 4-fold difference. Of note, GRL-0519's EC₅₀s with HSA or AAG (1.9 to 2.4 nM) significantly exceeded those of the four FDA-approved PIs (Table 4).

In vitro selection of HIV-1 variants resistant to GRL-0519.

We next attempted to select HIV-1 variants resistant to GRL-0519 by propagating a laboratory HIV-1 strain, HIV-1_{NL4-3}, in MT-4 cells in the presence of increasing concentrations of GRL-0519 as previously described (24). HIV-1_{NL4-3} was initially exposed to 0.0007 μM GRL-0519 and underwent 37 passages, after which the concentration of GRL-0519 was found to have increased 19-fold (0.0131 μM) compared to that at the initiation of selection. Judging from the amounts of p24 Gag protein produced in the culture medium (up to ~282 ng/ml), the replicative capacity of HIV-1_{NL4-3} at passage 37 (HIV₅₁₉^R_{P37}) was thought to have been reasonably well maintained. Compared to the kinetics of the emergence of variants resistant to APV, the emergence of GRL-0519- and DRV-resistant variants was substantially delayed (Fig. 2). Of note, HIV-1 variants resistant to APV and capable of replicating at >5 μM emerged by passage 20, and variants resistant to GRL-0529, replicating at >1 μM, emerged by passage 6, while it became fairly difficult to increase the concentrations of DRV and GRL-0519 around and beyond passage 20, since the virus populations ceased to replicate with further increased concentrations.

The protease-encoding region of the proviral DNA isolated from infected MT-4 cells was cloned and sequenced at passages 6, 13, 22, 29, and 37 under GRL-0519 selection. The sequences of the region cloned and the percent frequency of identical sequences at each passage are depicted in Fig. 3. By passage 13, the wild-type protease gene sequence was seen in 7 of 19 clones, although an N37S substitution was noted in 10 of the 19 clones. However, by passage 22 and beyond, N37S disappeared, and the virus had mostly acquired K43I and A71T substitutions. As the passages proceeded, greater numbers of amino acid substitutions emerged. At passage 29, V82I substitution was seen in 16 of 22 clones, and V82I became dominant by passage 37 (25 of 26 clones). An L33V substitution was also observed in 4 of the 26 clones by passage 37. Reportedly, APV-resistant HIV-1 variants contain V32I, I50V, I54L/M, L76V, I84V, and L90M substitutions (30, 31), and such substitutions were also identified in the present study (data not

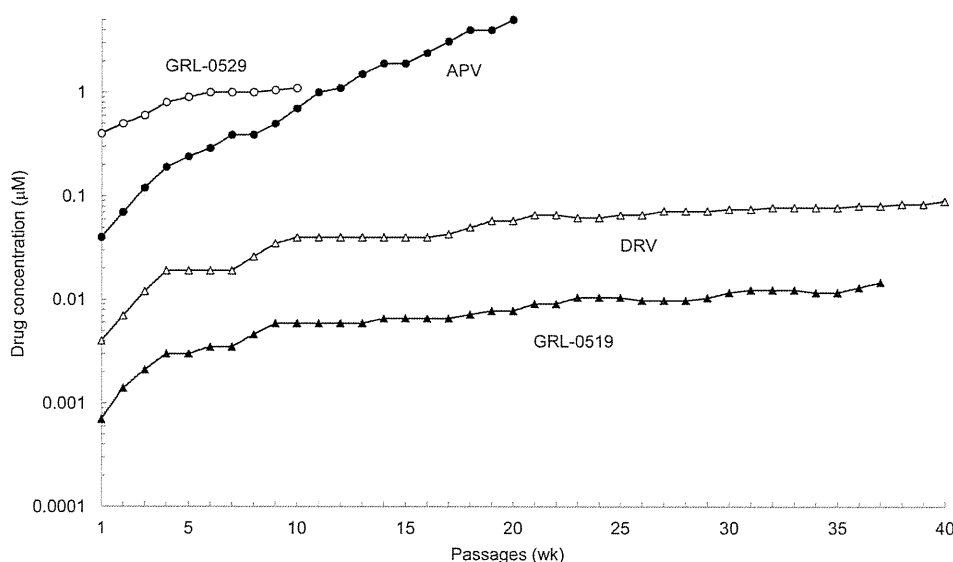


FIG 2 *In vitro* selection of PI-resistant HIV-1 variants. HIV-1_{NL4-3} was propagated in MT-4 cells in the presence of increasing concentrations of amprenavir (●), darunavir (△), GRL-0519 (▲), or GRL-0529 (○). Each passage of virus was conducted in a cell-free manner.

	10	20	30	40	50	60	70	80	90	99		
pNL4-3 PR	PQITLWQRPL	VTIKIGGQLK	EALLDTGADD	TVLEEMNLPG	RWKPKMIGGI	GGFIKVRQYD	QILIEICGHK	AIGTVLVGPT	PVNIIGRNLL	TQIGCTLNF		
6P-1		12/16
6P-2S.....		1/16
6P-3S.....		1/16
6P-4R.....		1/16
6P-5V.....A.....		1/16
13P-1S.....		8/19
13P-2		7/19
13P-3P.....		1/19
13P-4P.....S.....		1/19
13P-5S.....L.....		1/19
13P-6G.....		1/19
22P-1I.....T.....		10/14
22P-2I.....M.....T.....		1/14
22P-3S.....I.....T.....		1/14
22P-4V.....T.....		1/14
22P-5I.....T.....A.....		1/14
29P-1I.....T.....I.....		11/22
29P-2I.....T.....		3/22
29P-3T.....I.....T.....L.....		1/22
29P-4I.....T.....I.....C.....		1/22
29P-5I.....T.....A.....		1/22
29P-6I.....I.....T.....I.....		1/22
29P-7I.....T.....I.....T.....		1/22
29P-8M.....I.....T.....I.....		1/22
29P-9G.....I.....T.....I.....S.....		1/22
29P-10I.....T.....T.....		1/22
37P-1I.....T.....I.....		17/26
37P-2V.....I.....T.....I.....		3/26
37P-3I.....V.....T.....I.....		1/26
37P-4I.....T.....I.....N.....		1/26
37P-5V.....C.....T.....L.....		1/26
37P-6I.....T.....I.....A.....		1/26
37P-7A.....I.....T.....I.....A.....Y.....		1/26
37P-8V.....I.....T.....I.....		1/26

FIG 3 Amino acid sequences of the protease-encoding regions of HIV-1_{NL4-3} variants selected in the presence of GRL-0519. The amino acid sequences of protease, deduced from the nucleotide sequence of the protease-encoding region of each proviral DNA isolated at each indicated time, are shown. The amino acid sequence of the wild-type HIV-1_{NL4-3} protease is illustrated at the top as a reference.

shown). However, no such APV resistance-associated amino acid substitutions emerged during the GRL-0519 selection (Fig. 3). Additionally, HIV-1 selected with GRL-0519 (HIV₅₁₉^R_{P37}) acquired the following Gag amino acid substitutions: E17K and V84A in the matrix (MA) region and G61E and D152N in the CA region.

HIV₅₁₉^R_{P37} fails to replicate at a low concentration of GRL-0519. Since the replicative capacity of HIV₅₁₉^R_{P37} was thought to

be reasonably well maintained despite the presence of GRL-0519, as mentioned above, we determined the replication kinetics of HIV₅₁₉^R_{P37} and HIV-1_{NL4-3}. As shown in Fig. 4A, HIV-1_{NL4-3} failed to replicate in the presence of as little as 0.005 μM GRL-0519 during the entire culture period of 7 days. However, HIV₅₁₉^R_{P37} was capable of replicating in the presence of 0.005 and 0.01 μM GRL-0519, and the amount of p24 produced in the culture

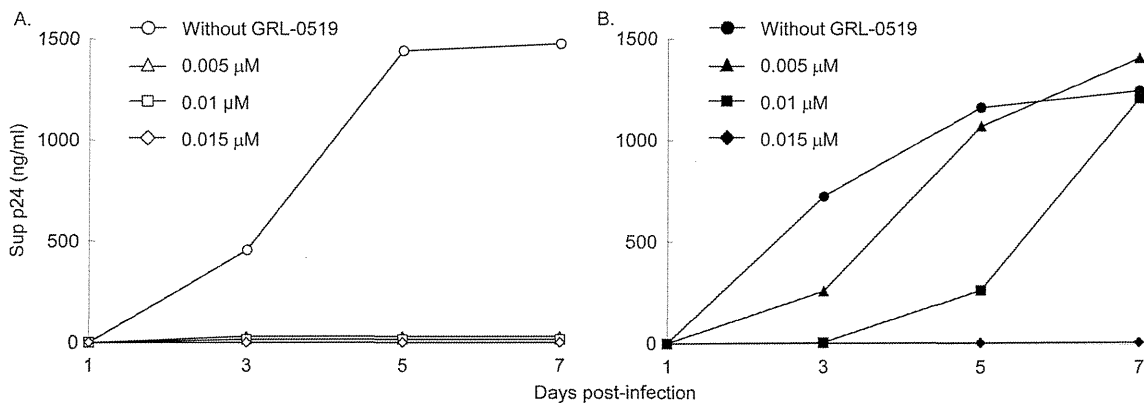


FIG 4 Replication kinetics of HIV-1_{NL4-3} and HIV₅₁₉^R_{P37}. MT-4 cells (3.2×10^5) were exposed to a HIV-1_{NL4-3} or HIV-1₅₁₉^R_{P37} preparation containing 10 ng/ml p24 in 6-well culture plates for 3 h, and the MT-4 cells were washed with fresh medium and divided into 4 fractions, each cultured with or without GRL-0519 (final concentration of MT-4 cells, 10^4 /ml; drug concentrations, 0, 0.005, 0.01, and 0.015 μM). The amount of p24 in each culture flask was measured every 2 days for up to 7 days, once at each time point.

TABLE 5 Roles of 3 amino acid substitutions that emerged during selection with GRL-0519 on GRL-0519's anti-HIV-1 activity

Virus ^a	GRL-0519 EC ₅₀ (nM) ^b
HIV-1 _{NL4-3}	0.5 ± 0.1
HIV-1 _{519^RP37}	6.0 ± 1.4 (12)
HIV-1 _{K43I}	1.2 ± 0.2 (2)
HIV-1 _{A71T}	1.4 ± 0.6 (3)
HIV-1 _{V82I}	3.2 ± 0.1 (6)

^a HIV-1_{K43I}, HIV-1_{A71T}, and HIV-1_{V82I} were created using a HIV-1_{NL4-3} plasmid clone.

^b The EC₅₀ values were determined with MT-4 cells, employing a p24 assay. The data shown represent mean values (±1 SD) derived from the results of two or four independent experiments. The numbers in parentheses represent the fold changes of EC₅₀s compared to the value against HIV-1_{NL4-3}.

medium reached the amount without GRL-0519 by day 7. However, HIV_{519^RP37} failed to replicate in the presence of 0.015 μM GRL-0519, and no p24 was detected throughout the culture period (Fig. 4B).

Roles of 3 amino acid substitutions that emerged during selection with GRL-0519 in GRL-0519's anti-HIV-1 activity. We also examined the roles of 3 substitutions that emerged during the GRL-0519 selection experiment, K43I, A71T, and V82I, using single-amino-acid substitutions carrying HIV-1_{NL4-3} variants. First, we determined the exact EC₅₀ of GRL-0519 against HIV_{519^RP37} using a p24 assay. GRL-0519 suppressed the replication of HIV_{519^RP37} with an EC₅₀ of 6.0 nM, 12-fold different from the EC₅₀ against HIV-1_{NL4-3} (Table 5). Next, we determined EC₅₀s of GRL-0519 against 3 single-amino-acid substitution-containing HIV-1_{NL4-3} variants (HIV-1_{K43I}, HIV-1_{A71T}, and HIV-1_{V82I}). GRL-0519 inhibited the replication of HIV-1_{K43I}, HIV-1_{A71T}, and HIV-1_{V82I} with EC₅₀s of 1.2, 1.4, and 3.2 nM, respectively (differences were 2-, 3-, and 6-fold compared to those against HIV-1_{NL4-3}, respectively) (Table 5).

In vitro selection of a mixture of 8 HIV-1_{MDR} variants resistant to GRL-0519. As described above and as shown in Fig. 2, the emergence of HIV-1 variants resistant to GRL-0519 was much delayed, and it took as many as 37 passages (37 weeks) for the virus to acquire its replicative activity in the presence of low concentrations (~0.01 μM). Thus, we attempted to select out GRL-0519-resistant variants using a mixture of 8 multidrug-resistant clinical isolates (HIV-1_{MDR}) as a starting HIV-1 population, as previously described (28, 32). We performed an additional selection experiment with MT4 cells and a mixture of 8 HIV-1_{MDR} variants as a starting virus population, with APV, DRV, and GRL-0519 and -0529. Similar to the results of selection experiments using MT-4 cells and HIV-1_{NL4-3}, the emergence of HIV-1 strains capable of replicating in the presence of GRL-0519 was significantly delayed compared to the cases with APV and DRV (Fig. 5).

Structural analyses of GRL-0519 interactions with protease.

We have recently reported the crystal structure depicting the binding mode and interactions of GRL-0519 with protease (17). GRL-0519 has a *tris*-THF moiety as its P2 ligand, and DRV has a *bis*-THF group as its P2 ligand (Fig. 1). Another difference is that GRL-0519 has a methoxybenzene as a P2' ligand, whereas DRV has an aniline. We analyzed the crystal structure of GRL-0519 with protease and compared it with the structure of protease complexed with DRV. We found that the oxygens of the first and second THF rings of GRL-0519 form hydrogen bond interactions with the backbone amide nitrogens of Asp-29 and Asp-30 in the S2

binding pocket of the protease (Fig. 6A). The urethane NH of GRL-0519 forms a hydrogen bond with Gly-27. The hydroxyl group of GRL-0519 forms hydrogen bonds with the catalytic Asp-25 and Asp-25'. The carbonyl oxygen and the sulfonamide oxygen of GRL-0519 form polar interactions with Ile-50 and Ile-50' in the protease flap through a bridging water molecule. These interactions are also seen for DRV (Fig. 6B). In fact, the polar interactions with Gly-27, Ile-50, and Ile-50', as well as with Asp-25 and Asp-25', are also seen for other protease inhibitors (33, 34). The oxygen of the methoxybenzene (P2' ligand) of GRL-0519 forms a hydrogen bond with the backbone amide nitrogen of Asp-30' in the S2' site of protease. This interaction at the S2' site is different in the case of DRV. DRV has an aniline as the P2' ligand, and it forms polar interactions with the backbone carbonyl oxygen of Asp-30'. The third THF ring of the *tris*-THF has water-mediated interactions with Thr-26 and Arg-8 involving 6 bridging water molecules. We also analyzed the van der Waals interactions between GRL-0519 and protease (Fig. 7). The methoxy moiety of GRL-0519 makes good van der Waals contacts with the Asp-29' and Asp-30' residues at the S2' site of protease (Fig. 7A). The contacts seem stronger than the interactions of the corresponding amine group of DRV (Fig. 7B). Analysis of the crystal structure of protease complexed with DRV had suggested that there is room

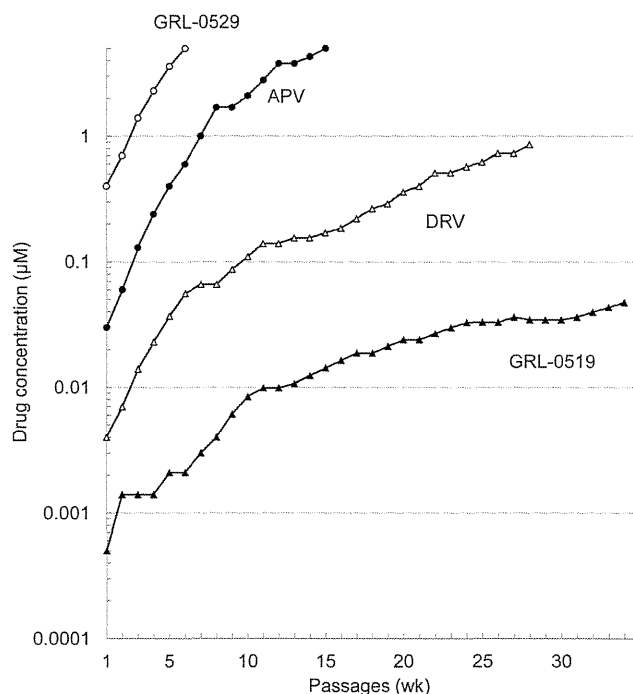


FIG 5 *In vitro* selection using a mixture of 8 multi-PI-resistant HIV-1_{MDR} isolates. A mixture of 8 different multi-PI-resistant clinical HIV-1 isolates was propagated in MT-4 cells in the presence of increasing concentrations of amprevir (●), darunavir (△), GRL-0519 (▲), or GRL-0529 (○). Each passage of virus was conducted in a cell-free manner. The amino acid substitutions identified in the protease-encoding regions of 2 different multi-PI-resistant clinical isolates compared to the consensus type B sequence cited from the Los Alamos database include L10I, I15V, E35D, N37E, K45R, I54V, L63P, A71V, V82T, L90M, I93L, and C95F in HIV-1_{MDR/A} and L10R, N37D, M46I, I62V, L63P, A71V, G73S, V77I, V82T, L90M, and I93L in HIV-1_{MDR/SS}. The amino acid substitutions of the other 6 HIV-1_{MDR} variants are given in Table 3, note a.

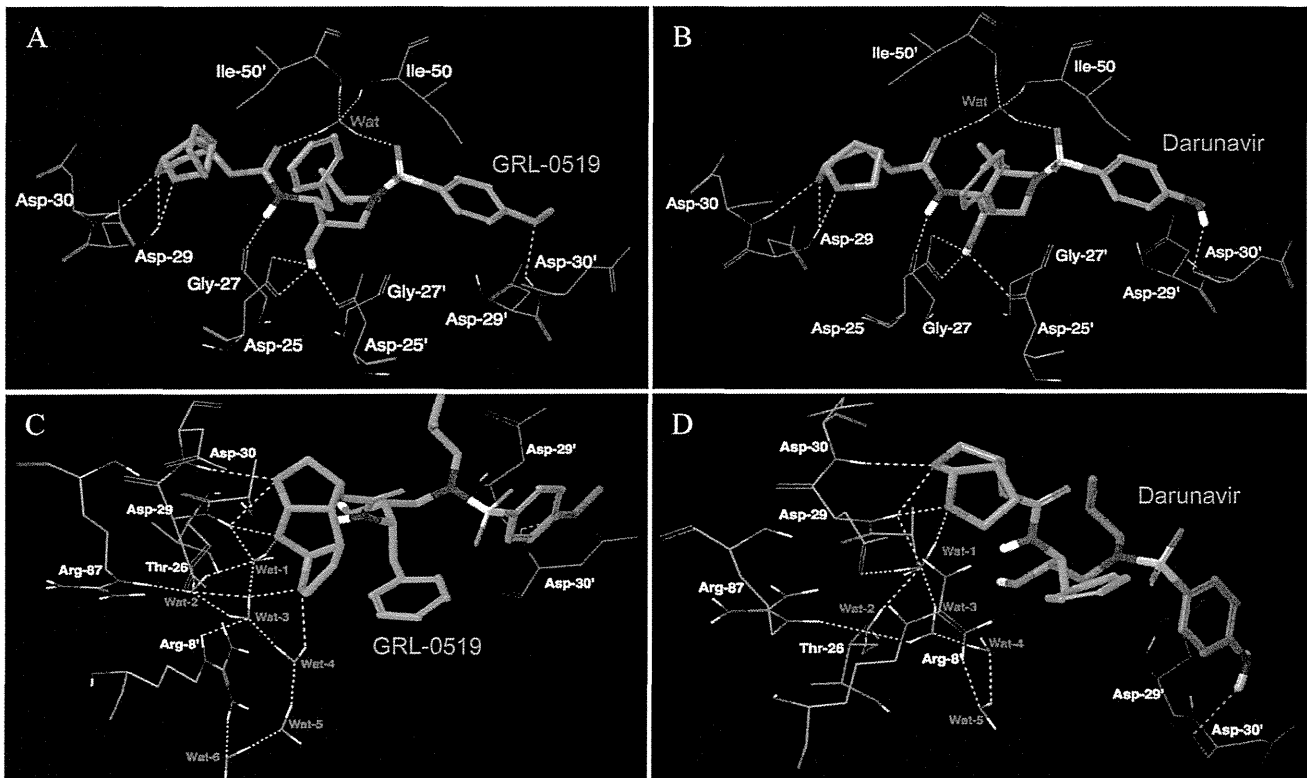


FIG 6 Hydrogen bond interactions of GRL-0519 and darunavir with protease. The interactions between protease–GRL-0519 (PDB ID, 3OK9) and protease–DRV (PDB ID, 2IEN) complexes as determined from their respective crystal structures were analyzed. (A and B) Similar interactions between protease–GRL-0519 (A) and protease–DRV (B) complexes. GRL-0519 has polar interactions with Asp-29, Asp-30, Gly-27, Asp-25, and Asp-25'. It has polar interactions with Ile-50 and Ile-50' through a bridging water molecule. The protease–DRV complex also has these polar interactions. Both inhibitors have polar interactions with different backbone atoms of Asp-30' in the S2' site of the protease. GRL-0519 interacts with the backbone amide nitrogen, whereas DRV interacts with the carbonyl oxygen. (C and D) The interactions of *tris*-THF (C) and *bis*-THF (D) moieties with water molecules highlight differences in the polar interactions of the two inhibitors. The additional THF ring of GRL-0519 interacts with an extra water molecule and has four more polar interactions than DRV. The figures shown here were made with Maestro version 9.3 (Schrödinger, LLC, New York, NY, 2012).

for an extra THF moiety to make additional contacts with protease. The crystal structure examined in the present study confirms that the *tris*-THF fully occupies the binding cavity and forms better van der Waals contacts with protease than the *bis*-THF of DRV (Fig. 7C and D). Contact (C) is defined by the following formula: $C = D_{12}/(R_1 + R_2)$, where D_{12} is the distance between atoms 1 and 2 and R_1 and R_2 are the van der Waals radii of atoms 1 and 2. A good contact is defined as follows: $1.30 > C > 0.89$.

The *tris*-THF moiety increases water-mediated polar interactions with protease. We further analyzed the polar interactions around the *tris*-THF ring of GRL-0519. The crystal structure shows that there are several water molecules that form polar interactions with GRL-0519 and bridge polar interactions with protease (Fig. 6C). Three water molecules, Wat-1, Wat-2, and Wat-3, form a tight network of hydrogen bonds among themselves and mediate polar interactions between GRL-0519 and the active-site residues. Wat-1 directly bridges the polar interactions between the oxygen of the second THF of GRL-0519 and Asp-29. The oxymethyl oxygen of the third THF ring of GRL-0519 enhances the strength of these networks of hydrogen bonds by its polar interactions with Wat-3 and Wat-4 (Fig. 6C). Wat-2 and Wat-3 also bridge the polar interaction between the oxymethyl of the third THF and Arg-87. There is one polar interaction between the third

oxymethyl and Arg-8' through Wat-3 and another network of polar interactions through Wat-4, Wat-5, and Wat-6. The crystal structure of DRV indicates the presence of five water molecules in this region compared to six present for GRL-0519 (Fig. 6D). The *tris*-THF of GRL-0519 and the additional water molecule are responsible for forming a total of 18 hydrogen bonds in this region compared to 14 for DRV, which has a *bis*-THF group as its P2 ligand.

DISCUSSION

GRL-0519, which contains a unique cyclic ether-derived nonpeptide P2 ligand, *tris*-THF, and a sulfonamide isostere, suppressed the replication of a wide spectrum of wild-type HIV-1 and HIV-2 strains with extremely low EC_{50} s (Table 1). GRL-0519 was highly potent against a variety of multidrug-resistant clinical HIV-1 isolates with EC_{50} s ranging from 0.0008 to 0.0034 μ M, while the existing FDA-approved PIs examined either failed to suppress the replication of those isolates or required much higher concentrations for viral inhibition (Table 3). GRL-0519 also efficiently blocked, with an EC_{50} of 0.03 μ M, the replications of a highly DRV-resistant variant (HIV_{DRV}^R_{P20}), to which the three PIs (APV, ATV, and LPV) had EC_{50} s of >1 μ M and DRV had an EC_{50} of 0.255 μ M (Table 3). Moreover, GRL-0519 exerted potent activity

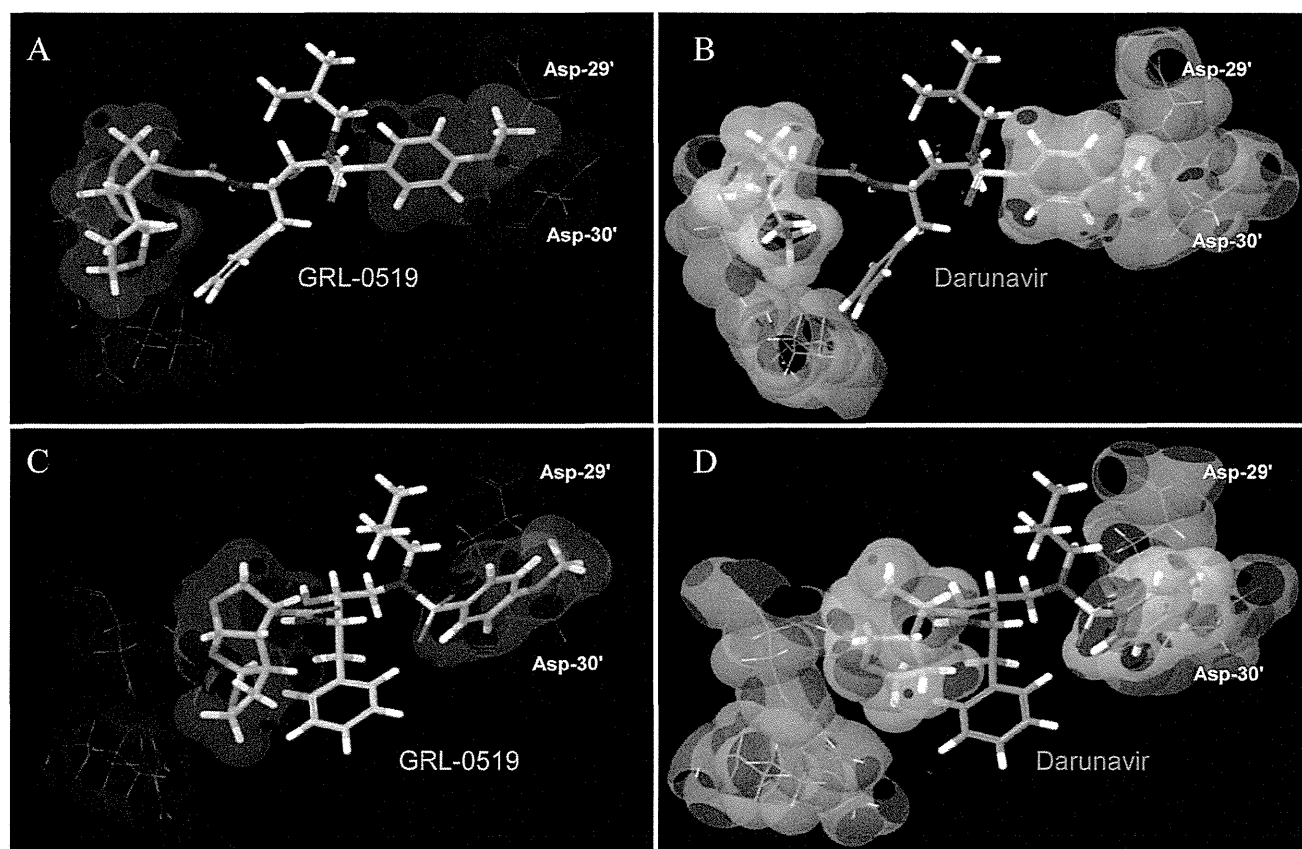


FIG 7 van der Waals interactions of GRL-0519 and darunavir with protease. GRL-0519 is shown as sticks (green carbons), and the van der Waals surfaces of selected moieties of GRL-0519 and its complexed protease are shown in gray and blue, respectively. DRV is shown as sticks (gray carbons), and the van der Waals surfaces of selected moieties of DRV and its complexed protease are shown in yellow and plum, respectively. (A and B) van der Waals interactions between the benzomethoxy of GRL-0519 and Asp-29' and Asp-30' of protease (A) and interactions between the aniline of DRV and Asp-29' and Asp-30' of protease (B). The surface interactions suggest that the methoxy group of GRL-0519 makes stronger van der Waals interactions with Asp-29' and Asp-30' of protease than does DRV. (C and D) The molecules are rotated to show the van der Waals surface interactions of the *tris*-THF (C) and *bis*-THF (D) moieties with protease. The *tris*-THF group of GRL-0519 forms better van der Waals contacts at the S2 site of protease than the *bis*-THF of DRV. The figures were made with Maestro version 9.3 (Schrödinger, LLC, New York, NY, 2012).

against laboratory PI-selected HIV-1 variants (except HIV-1_{APV}^R_{5 μ M}) with significantly low EC₅₀s (Table 2). GRL-0519 was less potent against HIV-1_{APV}^R_{5 μ M} with an EC₅₀ of 38.0 nM (a 76-fold difference), presumably due to the structural resemblance between GRL-0519 and APV, both of which contain a sulfonamide isostere (Fig. 1).

In an attempt to explain why GRL-0519 showed such potent activity against both wild-type and drug-resistant variants, we analyzed the crystal structure of the protease–GRL-0519 complex with protease (PDB ID, 3OK9). GRL-0519 has strong polar interactions with multiple regions of protease (Fig. 6A and C). The oxygens from two of the THF rings of GRL-0519 have strong polar interactions with the backbone amide nitrogens of Asp-29 and Asp-30. GRL-0519 also forms hydrogen bonds with Gly-27 and with the side chains of the catalytic aspartates, Asp-25 and Asp-25'. The sulfonamide oxygen and the carbonyl oxygen form polar contacts with Ile-50 and Ile-50' in the flap through the bridging water molecule. Comparison of the crystal structures of protease complexes with GRL-0519 and protease complexes with DRV highlights the similarities and differences in interactions between these two inhibitors. The two oxygens from the *bis*-THF of DRV

form hydrogen bond interactions with the backbone amide nitrogens of Asp-29 and Asp-30 (Fig. 6B). DRV also forms polar interactions with Gly-27, Asp-25, and Asp-25' and polar contacts with the protease flap through the bridging water molecule. However, there are important differences in the interactions of GRL-0519 and DRV with protease. An additional water molecule (Wat-3 in Fig. 6C) around the *tris*-THF ring of GRL-0519 is observed in the crystal structure. This water molecule forms hydrogen bond interactions with the third THF ring of GRL-0519 and enhances the polar contact through a network of hydrogen bonds with Asp-29, Thr-26, Arg-87, and Arg-8'. There are four additional polar contacts arising out of the presence of the third THF ring of GRL-0519 and Wat-3 compared to the water-mediated polar interactions in this region of the protease complexes with DRV (Fig. 6D). Even though both GRL-0519 and DRV form polar interactions with the backbone atoms of Asp-30' in the S2' site, they interact with different sets of atoms (Fig. 6A and B). GRL-0519 forms the hydrogen bond with the amide nitrogen, whereas DRV forms polar contact with the carbonyl oxygen of Asp30'. The van der Waals surface interactions of GRL-0519 and DRV are also different (Fig. 7A to D). The *tris*-THF and methoxy group of GRL-0519 form

stronger van der Waals contacts with the S2 and S2' sites of protease, respectively. These interactions are stronger than the corresponding interactions of the *bis*-THF and aniline groups of DRV.

It should be noted that GRL-0519, as previously reported (35), blocks the dimerization of HIV-1 protease monomer subunits more potently, by at least 10-fold, than DRV, as examined by a fluorescence resonance energy transfer (FRET)-based HIV-1 expression assay that uses cyan (CFP) and yellow (YFP) fluorescent protein-tagged protease monomers (17). Considering that the dimerization of HIV-1 protease subunits is an essential process for its acquisition of proteolytic activity, which plays a critical role in the maturation and replication of the virus (36), the potent activity of GRL-0519 to block protease dimerization should also contribute to the greater antiviral potency of GRL-0519 than of DRV. Taken together, the stronger polar and nonpolar contacts of GRL-0519 with protease, as observed in its crystal structure, in addition to its potent activity to block protease dimerization, are likely to be responsible for its much more potent antiviral activity than that of DRV.

In our study, all the PIs examined showed no significant reduction of antiviral activity with the addition of HSA (Table 4). In contrast, the addition of AAG substantially reduced the antiretroviral activities of APV, ATV, and DRV by more than 9-fold, and their EC₅₀s increased to 286.3, 32.7, and 33.8 nM, respectively. However, the reduction with GRL-0519 was only 4-fold, and its absolute EC₅₀ was as low as 2.4 nM (Table 4). AAG is an acute-phase protein, and its concentration can increase upon injury, surgery, inflammation, malignancy, and infection, including HIV-1 infection (29, 37). Therefore, this feature of GRL-0519 may represent an advantage for its potential clinical application.

In our HIV-1_{NL4-3} selection experiment using GRL-0519, the emergence of GRL-0519-resistant variants was substantially delayed compared to other PIs and DRV. The use of a mixture of multiple-drug-resistant HIV-1 isolates can expedite the emergence of variants resistant to the drug used for the selection *in vitro* through homologous recombination and should reflect what occurs within individuals harboring a number of drug-resistant HIV-1 species (quasispecies) (28, 32). Thus, in this study, we also employed the mixture of 8 different multi-PI-resistant clinical isolates as a starting viral population (Fig. 5). In the present study, by passage 37, 3 major amino acid substitutions (K43I, A71T, and V82I) were identified in PR of HIV-1_{NL4-3}. The residue V82 is located in the vicinity of the binding pocket of the protease and forms van der Waals contact with GRL0519. The V82A substitution is reportedly associated with resistance against various protease inhibitors (33, 34). However, K43 and A71 are distal from the inhibitor binding pocket and have no direct association with GRL0519. Thus, it is likely K43I and A71T are secondary substitutions. It is particularly noteworthy that we failed to select the A28S amino acid substitution in the present selection experiment with GRL-0519. In our previous studies of potent PIs, such as TMC-126 and GRL-1398, containing a paramethoxy group in the P2' site, the A28S amino acid substitution was identified as a resistant variant (19, 25). In this regard, the combination of *tris*-THF as the P2 ligand and the paramethoxy moiety at P2' seems to have prevented the selection of the A28S substitution as a resistant variant.

The *tris*-THF moiety has more interactions with the S2 site of the protease than the *bis*-THF present in either TMC-126 or DRV. Also, the *p*-OCH₃ moiety seems to have more favorable van

der Waals interactions with the S2 site than DRV (Fig. 7). It would be reasonable to expect that the combination of *tris*-THF and *p*-OCH₃ may increase the activity, but the antiviral data suggest that GRL-0519 and TMC-126 have essentially the same antiviral activity (see Table S1 in the supplemental material). For any potential antiretroviral agents (including protease inhibitors) to exert their antiretroviral activity even *in vitro*, multiple factors are involved. They include (i) structural stability in culture medium, as well as in the cytoplasm; (ii) permeability into cells; and (iii) compartmentalization. Moreover, such potential antiretroviral agents have to tightly bind to the active site of the target viral protein (i.e., the protease active site) but should not bind to cellular proteins critical to the survival and functionality of the cells. It is possible that while GRL-0519 has greater interactions derived from the presence of the *tris*-THF group, those interactions are not directly reflected in the ultimate antiretroviral activity.

Since the GRL-0519-selected variant, HIV₅₁₉^R_{P37}, was thought to be substantially replication competent, the replication kinetics of HIV-1_{NL4-3} and HIV₅₁₉^R_{P37} were compared in the presence and absence of GRL-0519. The data showed that HIV-1_{NL4-3} failed to replicate in the presence of 0.005 μM GRL-0519 and that HIV₅₁₉^R_{P37} did the same in the presence of 0.015 μM GRL-0519 throughout the 7-day culture period (Fig. 4). The concentration range of 0.005 to 0.015 μM is relatively easily achieved in the clinical use of various PIs. For example, the peak and nadir plasma levels of DRV were ~7.1 and 3.4 μM when 400 and 100 mg of DRV/RTV were administered twice daily for 7 days (38). Considering that the EC₅₀s of GRL-0519 are extremely low, ranging from 0.0005 to 0.030 μM (Tables 1 to 4), and that GRL-0519's selectivity index of 63,714 is highly favorable compared to other conventional PIs examined in this study (Table 1), both the anti-HIV potency and safety of GRL-0519 could be favorable, although the efficacy and emergence of adverse effects should be ultimately determined by controlled clinical trials.

In conclusion, GRL-0519 possesses a number of fairly favorable features for the development of the compound as a potential therapeutic for HIV-1 infection and AIDS. However, its oral bioavailability, pharmacokinetics/pharmacodynamics, and biodistribution remain to be determined, and further investigation is warranted.

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